Physiological, Enzymatic and Molecular Changes of *Acipenser schrencki* Induced by Heat Exposure

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Abstract **[Objective]** The paper was to understand the physiological, enzymatic and molecular changes of *Acipenser schrencki* induced by heat exposure. **[Method]** The effects of heat exposure on *A. schrencki* were investigated. The temperature was increased from (18± 0.5) ℃ by 0.08 ℃/min and respectively kept at (32±0.5) ℃ for 2 , 4 and 6 h. The respiration rate, related biochemical indicators and heat shock protein 70 (Hsp 70) mRNA expression were investigated. **[Result]** At (32±0.5) ℃, the activities of total protein, albumin, globulin, urea nitrogen, ceatinine, malondialdehyde, aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase fluctuated dramatically from 2 h to 6 h. Meanwhile, the respiration rate and Hsp 70 mRNA expression in heart, liver and spleen were significantly increased from 2 h to 6 h. In comparison, the lower respiration rate and tiny fluctuations in activities of biochemical indicators were consistent with the lower Hsp 70 expression. **[Conclusion]** During the process of heat stress, *A. schrencki* seems to be sensitive to such temperature, and manifests generally consistent gill breathing, biochemical indicators and Hsp70 gene expression, implying better heat tolerance.

Keywords *Acipenser schrencki*; Biochemical indicators; Heat exposure; Hsp70 mRNA

Stress responses are considered to be essentially adaptive or protective and thus should prevent or minimize detrimental effects of the stressor that is imposed upon the animal. Animals behave and respond differently to resist heat stress[1–2]. In fishes, one adaptational response for cooling the body is accelerating the respiration frequency, thus, the respiration rate has been confirmed to be a sensitive physiological indicator to measure the levels of discomfort/comfort to animals[3]. The increased respiration rate could lead to a higher gas exchange rate and would further lead to blood chemical activity. Recently, much efforts have been dedicated to the responses of biochemistry induces. Liu *et al.*[4] reported that the activities of superoxide dismutase (SOD), catalase (CAT), lysozyme (LZM) and pyruvate kinase (PK) declined, but there was no significant response for acid phosphatase (ACP) and alkaline phosphatase (AKP) under heat stress (except for CAT). Dominique *et al.*[5] confirmed that the activity of LDH would be inhibited under heat stress. In addition to those serum enzymes, there are also many other factors that might influence the reaction of serum proteins. Rao *et al.*[6] found that the concentration of total protein (TP) and albumin (ALB) under heat stress were all lower than that of control group, but these results were conflicted with other results[7]. However, there are so many different results about the blood biochemistry indices. The uncertainty parameters might cause confusion in the study of biochemistry and physiology response of heat-stressed fishes. On the other hand, the thermal requirement of fish changes at different growth periods and among varieties. So the biochemistry reaction of *Acipenser schrencki* to heat water environment will change. Additionally, higher temperature triggers the production of heat shock proteins (Hsps) family that protect the cells against harm to manage the environmental stress[8]. Among others, the Hsp70 protein was elevated in coho salmon exposed to thermal stress and the Hsp70 gene was also up-regulated when juvenile rainbow trout were exposed to high temperature[9]. These findings suggested that Hsp70 may act as a critical heat stress-associated factor in fish.

Although there are many studies conducted to evaluate the effects of heat stress on animals, the effect of heat water environment on *A. schrencki* have been overlooked and research works have been seldom conducted. The aim of this study was thus to test the adaptive responses of *A. schrencki* to high water temperature for 2–6 h and identify the suitable indicators, such as respiration rate, key blood indicators and Hsp 70 gene express in heart, liver and spleen under short-term heat stress and hopefully use them as markers for the evaluation of survivability under heat stress for short time. This study would also provide a scientific basis for understanding the mechanism of *A. schrencki* to heat tolerance.

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1 Materials and Methods

1.1 Experimental procedure All fishes (*A. schrencki*) were obtained from a local fish farm in Fuzhou of China, and identified by the species identification sheets released by *Food and Agriculture Organization*[10]. All fishes were acclimatized to the experimental conditions for 1 week before the start of the experiment. Healthy fishes of similar sizes [mean length (587.1±46.7) mm; mean weight (555.3 ±65.4) g] were transferred into circulating water systems consisting of several 130 cm×70 cm×80 cm breeding tanks. Fishes were kept in filtered seawater at (18±0.5) ℃ for a week while being fed commercial food designed for *A. schrencki* at 15:00 every day. During the rearing time, the dissolved oxygen was 6.0–7.0 mg/L, and the pH was 7.6–8.1. The photoperiod was 12 h light/12 h dark with light from 06:00 to 18:00. Approximately 70% of the water in the system was refreshed daily to minimize ammonia levels (0.25 mg/ L).

Eighty fishes were randomly divided into four groups (A, B, C and D). Groups B, C and D were the experimental groups and group A was designed as the control group. After 1 week acclimation in the previously described conditions, the temperatures in the tanks of groups A, B and C were ramped from (18±0.5)℃ to (32±0.5)℃ at a rate of 0.08℃/min and kept at (32±0.5)℃ for 2 h (group B), 4 h (group C) and 6 h (group D), respectively. The heating rate was similar to that experienced during the course of a day in this species' natural habitat [11]. The tank temperature in the group A was kept constant at (18 ±0.5) ℃. Twenty individuals from groups B, C or D were removed respectively at 2, 4 and 6 h at (32±0.5) ℃. Twenty individuals were also removed randomly from group A. About 70%–75% of the water in the system was refreshed by seawater of the same temperature every day to ensure good water quality. The gill respiration rate was monitored every 24 h to survey the fish's heath and repeated three times. All fishes used in this study were combined with the guide for the care and use of laboratory animals of China.

1.2 Sample collection All fishes were anesthetized with eugenol at the end of the experiment. Blood was collected from the tail vein for the measurement of blood indicators. Organs or tissues for Hsp70 gene expression analysis were saved or frozen in liquid nitrogen.

1.3 Measurement of serum biochemical indicators The concentration of the albumin (ALB), total protein (TP), globulin (GLO), urea nitrogen (UN), ceatinine (CRE), malondialdehyde (MDA) and the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) in the serum of treated and control fishes were assayed using test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) via a biochemical analyzer (VetTest 8008; Idexx Laboratories, Inc, Westbrook, ME, USA). The concentration of TP at 540 nm and ALB at 628 nm were measured by spectrophotometric and turbidimetric methods. The concentration of GLO (g/L) was calculated by the concentration difference of TP and ALB. The concentration of UN (mmol/L) was measured at 340 nm based on the oxidation of nicotinamide adenine dinucleotide hydrogen (NADH), and that of CRE (μmol/L) was measured at 505 nm by picric acid method. The concentration of MDA (mmol/L) was measured at 532 nm by thiobarbituric acid reactive substances assay kit. The activities of AST (IU/L) was assayed spectrophotometrically at 450 nm in a coupled reaction with malate dehydrogenase in the presence of NADH, that of ALT (IU/L) was assayed spectrophotometrically at 570 nm in a coupled reaction with alanine in the presence of NADH, and LDH activity (IU/L) was measured at 500 nm by 2,4-dinitrophenylhydrazine colorimetric reaction method. All the activities were calculated according the formulae shown in Fig.1. All determinations of biochemical indicators were repeated three times.

1.4 Quantitative real-time RT-PCR Total RNA was isolated from heart, liver and spleen samples using the Animal RNA Purification Reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was determined spectrophotometrically and its quality was verified by ethidium

$$\text{TP (g/L)}=\frac{OD_{tem}-OD_{com}}{OD_{not}}\times TP_{sta}\ \text{(g/L)}$$

$$\text{ALB (g/L)}=\frac{OD_{tem}-OD_{com}}{OD_{sta}-OD_{not}}\times ALB_{sta}\ \text{(g/L)}$$

$$\text{GLO (g/L)}=\text{TP (g/L)}-\text{ALB (g/L)}$$

$$\text{UN (mmol)}=\frac{OD_{tem}A1-OD_{tem}A2}{OD_{sta}A2-OD_{sta}A1}\times UN_{sta}\ \text{(mmol)}$$

$$\text{CRE (mol/L)}=\frac{OD_{tem}A2-OD_{tem}A2}{OD_{sta}A2-OD_{sta}A1}\times CRE_{sta}\ \text{(mol/L)}$$

$$\text{MDA (mmol/m L)}=\frac{OD_{tem}-OD_{com}}{OD_{sta}-OD_{not}}\times MDA_{sta}\ \text{(10 nmol/mL)}\times X$$

$$\text{AST (IU/L)}=\Delta A/\text{min}\times\frac{TV\times 1\ 000}{6.22\times SV\times P}$$

$$\text{ALT (IU/L)}=\Delta A/\text{min}\times\frac{TV\times 1\ 000}{6.22\times SV\times P}$$

$$\text{LDH (IU/L)}=\frac{OD_{tem}-OD_{com}}{OD_{sta}-OD_{not}}\times LDH_{sta}\ \text{(2 mmol/L)}\times X\times 1\ 000$$

Note: *A*1, 30 s; *A*2, 90 s; ALB, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CRE, ceatinine; *com*, control samples; *not*, blank solution; GLO, globulin; LDH, lactate dehydrogenase; MDA, malondialdehyde; *OD*, absorbance values; *P. cuvette* diameter (cm); *sta*, standard samples; *SV*, sample volume; *tem*, tested samples; TP, total protein; TV, total volume; UN, urea nitrogen.

Fig.1 Formulae used for calculating the concentration or activities of serum biochemical indicators

bromide staining in 1.2% agarose gel. Total RNA was treated with RNase-free DNase I (Invitrogen). About 1 μg of total RNA was used as a template in the first strand cDNA synthesis using MMLV RNase-H Reverse Transcriptase (Promega, Madison, Wisconsin, USA) that was performed according to the manufacturer's protocol. The optimal parameters for each gene are listed in Tab. 1. Quantitative real-time RT-PCR was performed using SYBR Premix Ex *Taq*TM (TaKaRa code: DRR041A, TaKaRa Biotechnology, Dalian, China) on a PCR System Mx 3000P (Stratagene, La Jolla, CA, USA) in accordance with the manufacturer's instructions. Briefly, 2 μL of 10-fold diluted cDNA was used in a final volume of 20 μL containing 10 μL of SYBR Green Real-Time PCR Master Mix (Thermo fisher scientific, USA) and 0.2–0.8 μM of sense and antisense primers for Hsp70. Fish β-actin mRNA was used as a reference gene for normalization. The quantitative real-time PCR conditions were as follows: denaturation at 95 ℃ for 30 s, followed by 45 cycles of 95 ℃ for 5 s, 62 ℃ for 34 s (fluorescent data collection) and repeated at least five times. The $2^{-\Delta\Delta Q}$ method was used to analyze the RT-PCR data (Livak and Schmittgen 2001). Mock RT and no-template controls were used to monitor for possible contamination of genomic DNA both in the RT reaction and during PCR. Pooled samples made by mixing equal quantities of total cDNA from all samples were used to optimize PCR conditions and to tailor the standard curves for each gene. Melting curves were performed to ensure that a single specific PCR product was amplified for each gene.

1.5 Data analysis All data were shown as X ± standard deviation. Results were statistically analyzed using one-way ANOVA, followed by Statistical Packages for the IBM SPSS (Ver.19.0). Different capital letters indicated extremely significant difference ($P<0.01$), and different lowercase letters indicated significant difference ($P<0.05$).

2 Results and Analysis

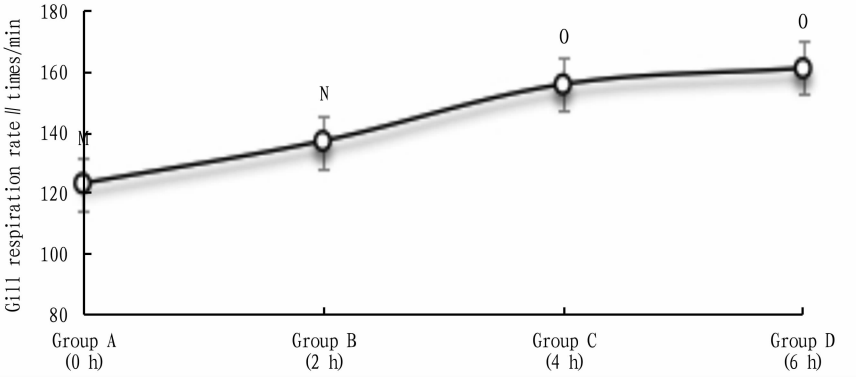
2.1 Respiration rate Before the sample collection, all fishes were still survival. As shown in Fig.2, the respiration rate of all fishes in the control group [group A, (18± 0.5) ℃] were kept at (122.90±7.24) times/min; when the temperature raised to (32± 0.5) ℃ and kept for 2 h, the respiration rates were significantly increased to (136.90 ±11.20) times/min (group B), (155.60±10.12) times/min for 4 h (group C) and (160.90 ±13.08) times/min for 6 h (group D). These data indicated that the respiration rates were significantly increased from 2 h to 6 h at (32±0.5) ℃.

2.2 Measurement of the serum biochemical indicators The activities of serum ALB, LDH, TP, GLO, UN, CRE, MDA, AST and ALT are shown in Tab. 2 and Fig.3. The data of experimental groups were compared with their own control groups. All the serum biochemical indicators activities fluctuated dramatically from 2 h to 6 h at (32±0.5) ℃. The

Tab. 1 The primers designed for gene expression

Gene name		Primer sequence (5′-3′)	Length of products// bp
Hsp 70	sense	ATTGCTTATGGCTTGGAC	130
	anti-sense	ACAGCGGTGGATTTCACTT	
β-actin	sense	GGTTTCGCTGGAGATGAT	99
	anti-sense	TGGGATACTTCAGGGTCAG	

Note: S. The sense primer; A. The anti-sense primer.



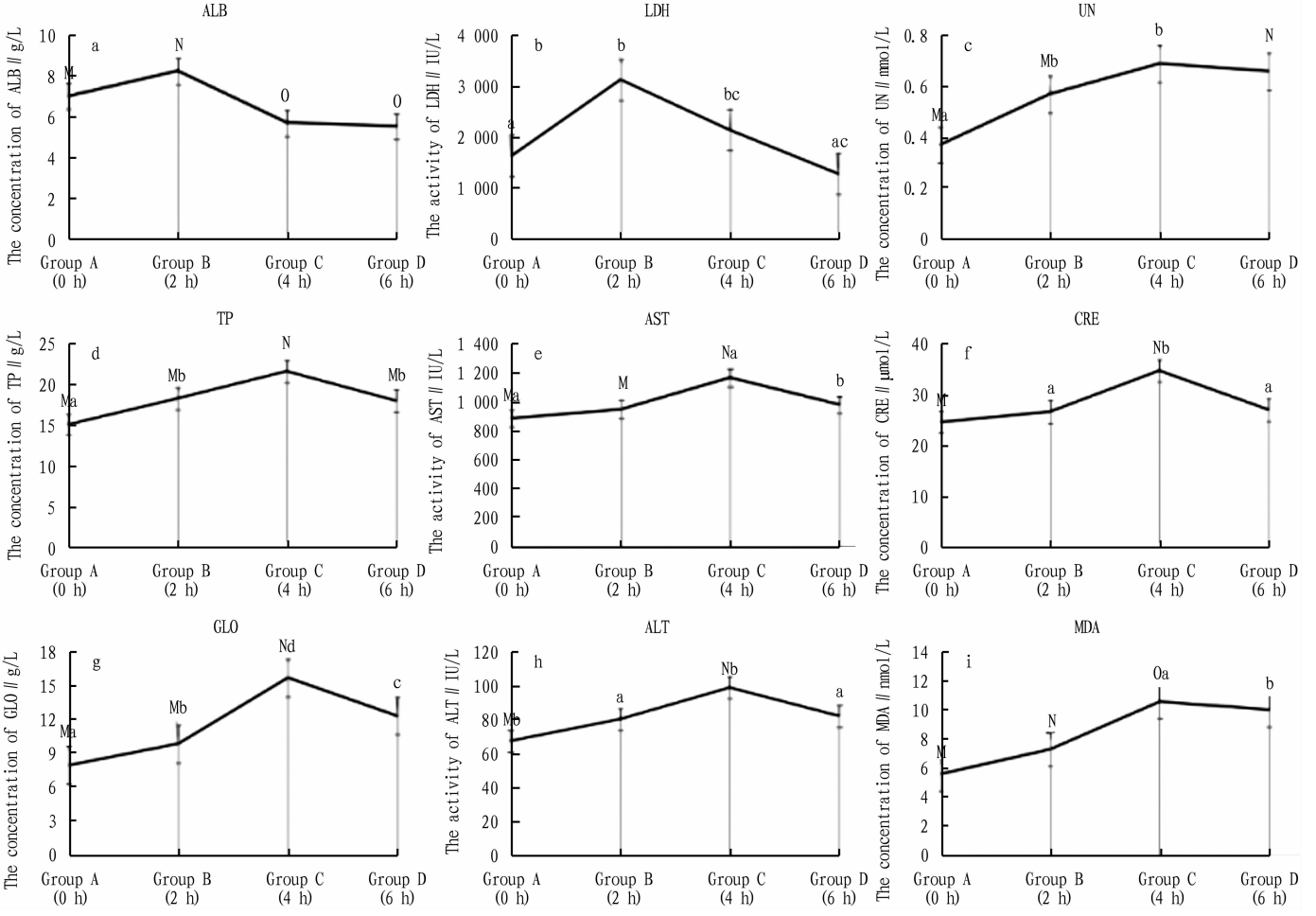
Note: Different capital letters represent extremely significant difference ($P<0.01$).

Fig.2 The gill respiration rate of fish in treatment groups and control group

Tab. 2 The concentrations of serum biochemical indicators in treatment groups and control group

Biochemical indicators	Groups			
	Group A (0 h)	Group B (2 h)	Group C (4 h)	Group D (6 h)
ALB // g/L	6.96±0.24^{M}	8.20±0.39^{N}	5.66±0.33^{O}	5.49±0.12^{O}
LDH // IU/L	1 652.00±371.90^{a}	3 141.78±611.80^{b}	2 157.35±568.44^{bc}	1 295.89±140.07^{ac}
UN // mmol/L	0.37±0.08^{Ma}	0.57±0.06^{Mb}	0.69±0.04^{b}	0.66±0.05^{N}
TP // g/L	14.88±0.76^{Ma}	18.04±0.60^{Mb}	21.34±0.62^{N}	17.77±0.88^{Mb}
AST // IU/L	881.29±21.57^{Ma}	942.20±52.69^{M}	1159.45±40.22^{Na}	975.16±47.13^{b}
GLO // g/L	7.93±0.98^{Ma}	9.84±0.70^{Mb}	15.67±0.50Nd	12.28±0.97^{c}
CRE // μmol/L	24.44±2.76^{M}	26.48±3.15^{a}	34.51±2.03^{Nb}	26.87±2.97^{a}
ALT // IU/L	67.25±8.54^{Mb}	80.10±4.12^{a}	98.72±8.88^{Nb}	81.93±7.04^{a}
MDA // nmol/L	5.57±0.26^{M}	7.25±0.34^{N}	10.49±0.43^{Oa}	9.95±0.20^{b}

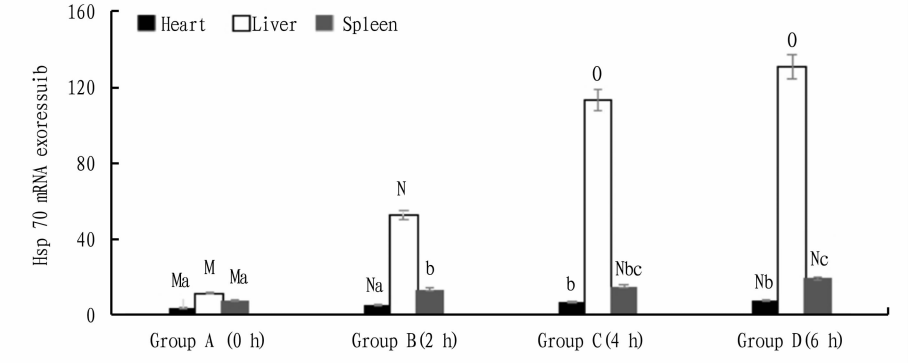
Note: Different capital letters in the same row represent extremely significant difference ($P<0.01$), and different lowercase letters represent significant difference ($P<0.05$). TP, total protein; ALB, albumin; GLO, globulin; UN, urea nitrogen; CRE, ceatinine; MDA, malondialdehyde; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase.



Note: Different capital letters represent extremely significant difference (P<0.01), and different lowercase letters represent significant difference (P<0.05). ALB, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CRE, ceatinine; GLO, globulin; LDH, lactate dehydrogenase; MDA, malondialdehyde; TP, total protein; TV, total volume; UN, urea nitrogen.

Fig.3 Measurement of serum biochemical indicators in treatment groups and control group

production of ALB and LDH activities were shown in similar manner. The ALB activity at (32± 0.5) ℃ was increased significantly at 2 h, then decreased from 4 h to 6 h, which peaked at 2 h (P<0.01). Analogously, the serum LDH activity at (32±0.5) ℃ was also peaked at 2 h, and decreased from 4 h to 6 h (P<0.05). Both the activities of ALB and LDH at 4 and 6 h were lower than its own controls (P< 0.05). All the activities of TP, GLO, UN, CRE, MDA, AST and ALT maintained at (32±0.5) ℃ were shown the similar manner and were all significantly higher than those of control groups, which increased from 2 h to 6 h and peaked at 4 h in treated groups (P<0.01).



Note: Different capital letters represent extremely significant difference (P<0.01), and different lowercase letters represent significant difference (P<0.05).

Fig.4 The relative quantitative expression of Hsp70 mRNA in heart, liver and spleen in treatment groups and control group

2.3 Hsp70 mRNA expression The quantitative real-time RT-PCR results showed that the expression of Hsp 70 mRNA in treated and control groups were shown in similar manner in the heart, liver and spleen tissues (Fig.4). Compared to its own control group, the expression of Hsp70 mRNA in all treated groups were significantly increased at (32±0.5) ℃ (P<0.01). The gene expression was increased over time at (32±0.5) ℃. All the most highest expression of Hsp70 mRNA occured at 6 h (heart, 7.67 ±1.84; liver, 130.61 ±21.33; spleen, 19.19±6.03), moderate at 4 h (heart,

6.50±1.94; liver, 113.17±2 0.55; spleen, 14.94±2.60) and lowest at 2 h (heat, 4.98±1.11; liver, 52.53±14.61; spleen, 13.56±4.34).

3 Discussion

Heat stress is a major cause of death in the aquatic industries. Fishes are more heat sensitive as the average consumption of fish has increased. During the heat stress, physiological and biochemical as well as molecular changes occurs in *A. schrencki* body which may directly or indirectly affect the survival of *A. schrencki*. This study systematically reports the changes of the respiration rates, biochemical indicators and Hsp 70 gene expression at (32±0.5) ℃ in *A. schrencki*, which will provide the basic understanding for the resistance mechanisms that allow *A. schrencki* to survive under heat stress.

Respiration rate could give an immediate response to the heat stress and appeared to be more sensitive indicator of heat stress than other physiological parameters[12], because increased respiration rate could contribute to evaporate heat loss accounting for 30% of the heat dissipation from the respiratory tract and is regarded as one of the primary mechanisms for maintenance of heat balance[13–14]. In this study, the respiration rates of *A. schrencki* were significantly increased in short time when water environment temperature was (32±0.5) ℃. Such phenomenon of significantly increased respiration rates of *A. schrencki* at (32±0.5) ℃ were consistent with that occurred in buffaloes and cattle in summer season[15] and may also contribute to vaporizing more heat. So, these significantly increased respiration rates indicated that *A. schrencki* were already overheated and tried to restore normal heat balance.

Blood biochemical indicators, such as serum proteins, body metabolites and enzymes, are key components of metabolic system that are closely linked to processes important for survival under heat stress. Short time heat stress increased protein catabolism to decrease plasma concentration of different protein and increase the contents of metabolites (Gonzalez-Esquerra and Leeson, 2005). The concentration of serum proteins and metabolites were closely linked to maintain the physiological balance for survival[16]. This study showed the concentration of serum proteins for TP, ALB and GLO were subsequently decreased after initially increased, and showed increased manner for UN,CRE and MDA when the water environment temperature raised to (32±0.5)℃. These results were partly consistent with the results of Han *et al.*[16]. The increasing contents of the metabolites for UN, CRE and MDA in serum could reflect the damage of heat exposure. Such diminishing manner of serum proteins and increasing levels of metabolites would contribute to increase the protein catabolism under heat stress, which is likely to produced glucose for sustaining life. Many studies have demonstrated a close link between the activities of AST, ALT and LDH critical to metabolic rate may be attributed to cellular damage as a direct consequence of heat stress for fishes[17–18]. In this context, the activities of serum AST, ALT and LDH rate at(32±0.5) ℃ were significantly higher than those of control groups. These results were not exactly match with the decreased AST activity and the unaffected ALT and LDH activities were found at high temperatures in previous study[19]. This apparent contradiction may be due to the different experimental conditions and fish species. However, other factors may contribute to the observed alterations in enzyme activities, since enzymes are complex and widely influenced. These comprehensive phenomena along with increase activities of serum enzymes at high temperature may suggest improved heat tolerance of *A. schrencki* at the expense of excessive blood damage. Therefore, those activities of AST, ALT and LDH that are highly correlated with the ability to withstand heat stress may serve as biochemical markers for the selection and development of *A. schrencki* more resistant to heat stress.

Although protein synthesis is disturbed under heat stress, it can not apply to heat shock proteins (HSPs). The HSPs family has been highly evaluated because the abundant expression of several members in the family under heat stress is predominately higher than in normal station. Among the cited HSP classes, Hsp 70 was correlated with the development of thermo-tolerance[20–22]. Transiently increased expression of Hsp 70 has been shown to be protective in many cultured cells and animals tissues from stress[23–24]. Previous report has described that the maximal rate of Hsp 70 synthesis occurs 3–5 h after heat shock and ceases after 8 h[25]. Here, a sharp increase of Hsp70 mRNA expression in the heart, liver and spleen tissues were observed from 2 h to 6 h at (32±0.5) ℃ and these results are partly line with previous studies mentioned above. Of course, the Hsp 70 levels under heat stress depend on cell and animal types. But, such increased levels of Hsp 70 mRNA should be at least accounting for the previous other results about the up-regulation of this protein during the early stages in other stress condition to overcome any environment injury. Therefore, all these results may collectively suggest that the induction of Hsp 70 gene after heat exposure for 2–6 h could be a danger signal raised in response to an exogenous injury and may provide ways to select heat stress-tolerant fishes with high production potential.

4 Conclusions

In brief, this study offers systematic evidence that heat exposure causes a series of well-characterized physiological changes in *A. schrencki* at (32±0.5) ℃. The generally consistence in the manifestation of respiration rate, enzyme activities and Hsp70 gene expression may be contributed to understand the mechanisms to heat tolerance and are also possible markers for the determination and selection of heat-resistant fishes.

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Conflict of interest

The authors declare that there have no conflicts of interest.

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